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(71) Applicant *(for all designated States except US)*: SMITHKLINE BEECHAM CORPORATION [US/US]; Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: LEVY, Mark, Alan [US/US]; 115 Reveille Road, Wayne, PA 19087 (US). GLEASON, John, Gerald [CA/US]; 8 Heron Hill Drive, Downington, PA 19335 (US).

(74) Agents: VENETIANER, Stephen et al.; SmithKline Beecham Corporation, Corporate Intellectual Property, UW2220, 709 Swedeland Road, P.O. Box 1539, King of Prussia, PA 19406-0939 (US).

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(57) Abstract

The present invention is to the novel compounds of Formula (I), their pharmaceutical compositions, and to the novel inhibition of ICE and ICE-like proteins for use in the treatment of apoptosis, and disease states caused by excessive or inappropriate cell death.

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Interleukin Converting Enzyme and Apoptosis

FIELD OF THE INVENTION

The present invention is to the discovery of a new method to block excessive or inappropriate apoptosis in a mammal.

BACKGROUND

It has been recognized for over a century that there are different forms of cell death. One form of cell death, necrosis, is usually the result of severe trauma and is a process that involves loss of membrane integrity and uncontrolled release of cellular contents, often giving rise to inflammatory responses. In contrast, apoptosis is a more physiological process that occurs in a controlled manner and is generally non-inflammatory in nature. For this reason apoptosis is often referred to as programmed cell death. The name itself (apoptosis: Greek for "dropping off", for example leaves from trees) implies a cell death that is part of a normal physiological process (Kerr et al., Br. J. Cancer, 26: 239-257 (1972)).

Apoptosis appears to be a carefully controlled series of cellular events which ultimately leads to death of the cell. This process for elimination of unwanted cells is active and requires expenditure of cellular energy. The morphological characteristics of apoptosis include cell shrinkage and loss of cell-cell contact, condensation of nuclear chromatin followed by fragmentation, the appearance of membrane ruffling, membrane blebbing and apoptotic bodies. At the end of the process, neighboring cells and macrophages phagocytose the fragments from the apoptotic cell. The process can be very fast, occurring in as little as a few hours (Bright et al., Biosci. Rep., 14: 67-82 (1994)).

The best defined biochemical event of apoptosis involves the orderly destruction of nuclear DNA. Signals for apoptosis promote the activation of specific calcium- and magnesium-dependent endonucleoases that cleave the double stranded DNA at linker regions between nucleosomes. This results in production of DNA fragments that are multiples of 180-200 base pair fragments (Bergamaschi et al., Haematologica, 79: 86-93 (1994); Stewart, JNCI, 86: 1286-1296 (1994)). When examined by agarose gel electrophoresis, these multiple fragments form a ladder pattern that is characteristic for most cells undergoing apoptosis.

There are numerous stimuli that can signal cells to initiate or promote cellular apoptosis, and these can be different in different cells. These stimuli can include glucocorticoids, TNFα, growth factor deprivation, some viral proteins, radiation and anticancer drugs. Some of these stimuli can induce their signals through a variety of cell surface receptors, such as the TNF / nerve growth factor family of receptors, which include CD40 and Fas/Apo-1 (Bright et al., supra). Given this diversity in stimuli that cause apoptosis it has been difficult to map out the signal transduction pathways and molecular factors involved in apoptosis. However, there is evidence for specific molecules being involved in apoptosis.

The best evidence for specific molecules that are essential for apoptosis comes from the study of the nematode C. elegans. In this system, genes that appear to be required for induction of apoptosis are Ced-3 and Ced-4. These genes must function in the dying cells and, if either gene is inactivated by mutation, cell death fails to occur (Yuan et al., Devel. Biol., 138: 33-41 (1990)). In mammals, genes that have been linked with induction of apoptosis include the proto-oncogene c-myc and the tumor suppresser gene p53 (Bright et al., supra; Symonds et al., Cell, 78: 703-711 (1994)).

In this critical determination of whether or not to undergo apoptosis, it is not surprising that these are genes that program for proteins that inhibit apoptosis. An example in C. elegans is Ced-9. When it is abnormally activated, cells survive that would normally die and, conversely, when Ced-9 is inactivated cells die that would normally live (Stewart, B.W., supra). A mammalian counterpart is bcl-2, which had been identified as a cancer-causing oncogene. This gene inhibits apoptosis when its product is overexpressed in a variety of mammalian cells, rendering them less sensitive to radiation, cytotoxic drugs and apoptotic signals such as c-myc (Bright et al., supra). Some virus protein have taken advantage of this ability of specific proteins to block apoptosis by producing homologous viral proteins with analogous functions. An example of such a situation is a protein produced by the Epstein Barr virus that is similar to bcl-2, which prevents cell death and thus enhances viral production (Wells et al., J. Reprod. Fertil., 101: 385-391 (1994)). In contrast, some proteins may bind to and inhibit the function of bcl-2 protein, an example being the protein bax (Stewart, B.W., supra). The overall picture that has developed is that entry into apoptosis is regulated by a careful balancing act between specific gene products that promote or inhibit apoptosis (Barinaga, Science, 263: 754-756 (1994).

Apoptosis is an important part of normal physiology. The two most often sited examples of this are fetal development and immune cell development. In development of the fetal nervous system, over half of the neurons that exist in the early fetus are lost by apoptosis during development to form the mature brain (Bergamaschi et al., Haematologica, 79: 86-93 (1994)). In the production of immune competent T cells

(and to a lesser extent evidence exists for B cells), a selection process occurs that eliminates cells that recognize and react against self. This selection process is thought to occur in an apoptotic manner within areas of immune cell maturation (Williams, G. T., J. Pathol., 173: 1-4 (1994); Krammer et al., Curr. Opin. Immunol., 6: 279-289 (1994)).

Dysregulation of apoptosis can play an important role in disease states, and diseases can be caused by both excessive or too little apoptosis occurring. An example of diseases associated with too little apoptosis would be certain cancers. There is a follicular B-cell lymphoma associated with an aberrant expression of functional bcl-2 and an inhibition of apoptosis in that cell (Bergamaschi et al., supra). There are numerous reports that associate deletion or mutation of p53 with the inhibition of apoptosis and the production of cancerous cells (Kerr et al., Cancer, 73: 2013-2026 (1994); Ashwell et al., Immunol. Today, 15: 147-151, (1994)). In contrast, one example of excessive or inappropriate apoptosis is the loss of neuronal cells that occurs in Alzheimer disease, possible induced by β-amyloid peptides (Barr et al., BioTechnology, 12: 487-493 (1994)). Other examples include excessive apoptosis of $CD4^+$ T cells that occurs in HIV infection, of cardiac myocytes during infarction / reperfusion and of neuronal cells during ischemia (Bergamaschi et al., supra); Barr et al., supra).

Some pharmacological agents attempt to counteract the lack of apoptosis that is observed in cancers. Examples include topoisomerase II inhibitors, such as the epipodophyllotoxins, and antimetabolites, such as ara-c, which have been reported to enhance apoptosis in cancer cells (Ashwell et al., supra). In many cases with these anti-cancer drugs, the exact mechanism for the induction of apoptosis remains to be elucidated.

In the last few years, evidence has built that ICE and proteins homologous to ICE play a key role in apoptosis. This area of research has been spurred by the observation of homology between the protein coded by Ced-3, a gene known to be critical for C. Elegans apoptosis, and ICE. These two proteins share 29% amino acid identity, and complete identity in the 5 amino acid portion thought to be responsible for protease activity (QACRG) (Yuan et al., Cell, 75: 641-652 (1993)). Additional homologies are observed between ICE and the product of the nedd-2 gene in mice, a gene suspected of involvement in apoptosis in the developing brain (Kumar et al., Genes Dev., 8: 1613-1626 (1994)) and Ich-1 and CPP32 (ICE and Ced-3 homolog-1), human counterparts of nedd-2 isolated from human brain cDNA libraries (Wang et al., Cell, 78: 739-750 (1994); Fernandes-Alnemiri et al., J. Biol. Chem., 269: 30761-30764 (1994)).

Further proof for the role of these proteins in apoptosis comes from transfection studies. Over expression of murine ICE caused fibroblasts to undergo programmed cell death in a transient transfection assay (Miura et al., Cell, 75: 653-660 (1993)). Cell death could be prevented by point mutations in the transfected gene in the region of greatest homology between ICE and Ced-3. As very strong support for the role of ICE in apoptosis, the authors showed that ICE transfection-induced apoptosis could be antagonized by overexpression of bcl-2, the mammalian oncogene that can prevent programmed cell death (Miura et al., supra). Additional experiments were performed using the crmA gene. This gene of the cowpox virus encodes a serpin protein, a family of proteins that are inhibitors of proteases (Ray et al., Cell, 69: 597-604 (1992)). Specifically, the protein of crmA has been shown to inhibit processing of pro-interleukin -1β by ICE. (Gagliardini et al. Science, 263: 826-828 (1994)) showed that microinjection of the crmA gene into dorsal root ganglion neurons prevented cell death induced by nerve growth factor deprivation. This result shows that ICE is involved in neuronal cell apoptosis. A more direct demonstration of ICE involvement comes from experiments in which ICE transfection is coupled with the co-expression of crmA, demonstrating a crmA-induced suppression of the ICE-induced apoptosis response (Miura et al., supra; Wang et al., supra).

In addition to ICE, researchers have examined the ability of ICE-like genes to promote apoptosis. (Kumar et al. supra) demonstrated that over expression of nedd-2 in fibroblasts and neuroblastoma cells resulted in cell death by apoptosis and that this apoptosis could also be suppressed by expression of the bcl-2 gene. Most recently, Wang et al., (Wang et al. , supra) examined the over expression of Ich-1 in a number of mammalian cells. Expression resulted in cell apoptosis, which could be antagonized by bcl-2 co-expression. Mutation of a cysteine residue, contained within the QACRG motif and presumed to be critical for protease function, to serine abolished apoptotic activity.

Further evidence for a role of a cysteine protease in apoptosis comes from a recent report by Lazebnik et al. (Nature, 371: 346-347 (1994)). These authors have used a cell-free system to mimic and study apoptosis. In their system there is a protease activity that cleaves the enzyme poly(ADP-ribose) polymerase at a site identical to a cleavage site in pre-interleukin-1β. However, this yet to be isolated protease and ICE appear to be different and to act on different substrate proteins. Blockade of protease activity in the system, using non-selective cysteine protease inhibitors, resulted in inhibition of apoptosis.

Taken together, the above evidence provides striking involvement of ICE and ICE-like proteins in the induction of apoptosis in mammalian cells. Brain interleukin-1 has been reported to be elevated in Alzheimer disease and Down syndrome (Griffin

et al., Proc. Natl. Acad. Sci. U. S. A., 86: 7611-7615 (1989)). There are also reports that interleukin-1 can increase the mRNA and production of β-amyloid protein, a major component of senile plaques in Alzheimer disease as well as in brains of people with Down syndrome and with aging (Forloni et al., Mol. Brain Res., 16: 128-134 (1992); Buxbaum et al., Proc. Natl. Acad. Sci. U. S. A., 89: 10075-10078 (1992); Goldgaber et al., Proc. Natl. Acad. Sci. U. S. A., 86: 7606-7610 (1989)). These reports can be viewed as additional evidence for the involvement of ICE in these diseases and the need for use of a novel therapeutic agent and therapy thereby.

To date, no useful therapeutic strategies have blocked excessive or inappropriate apoptosis. In one patent application, EPO 0 533 226 a novel peptide structure is disclosed which is said to be useful for determining the activity of ICE, and therefore useful in the diagnoses and monitoring of IL-1 mediated diseases. Therefore, a need exists to find better therapeutic agents which have non-toxic pharmacological and toxicological profiles for use in mammals. These compounds should block excessive or inappropriate apoptosis cells, and hence provide treatment for diseases and conditions in which this condition appears.

SUMMARY OF THE INVENTION

The present invention is to the novel compounds of Formula (I), their pharmaceutical compositions, and to the novel inhibition of ICE and ICE-like proteins for use in the treatment of apoptosis, and disease states caused by excessive or inappropriate cell death.

Another aspect of the present invention is to a pharmaceutical composition comprising a compound of Formula (I), or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier or diluent.

Another aspect of the present invention is to a method for the treatment of diseases or disorders associated with excessive IL-1β convertase activity, in a mammal in need thereof, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

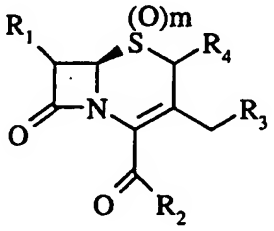
Another aspect of the present invention is to a method of preventing or reducing apoptosis in a mammal, preferably a human, in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

Another aspect of the present invention is to a method of blocking or decreasing the production of IL-1β and/or TNF, in a mammal, preferably a human, in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

DETAILED DESCRIPTION OF THE INVENTION

The compounds of the present invention may contain one or more asymmetric carbon atoms, in particular positions 6 and 7, and may exist in racemic and optically active forms. All of these compounds are included within the scope of the present invention. Preferably the compound has a 6R, 7S configuration.

Preferably the compounds of Formula (I) are represented by the structure:



(I)

wherein

R_1 is hydrogen, halogen, or an optionally substituted alkoxy;

R_2 is NR_aR_b;

R_a is hydrogen, or C_{1-4} alkyl;

R_b is C_{1-10} alkyl, optionally substituted arylC_{1-4} alkyl, optionally substituted heteroaryl C_{1-4} alkyl, optionally substituted C_{3-7} cycloalkyl, or R_a and R_b together with the nitrogen to which they are attached form a 5 to 10 membered ring which optionally contains an additional heteroatom selected from oygen, nitrogen or sulfur;

R_3 is hydrogen, -OC(O)R_5, or S(O)n R_6, or bromo;

R_4 is hydrogen, -OC(O)R_5, bromo or S(O)n R_6, provided that when R_3 is hydrogen, R_4 is other than hydrogen;

R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl or optionally substituted arylalkyl;

R_6 is optionally substituted aryl, or optionally substituted heteroaryl;

m is an integer having a value of 1 or 2;

n is 0, or an integer having a value of 1 or 2;

or a pharmaceutically acceptable salt thereof.

Suitably, for compounds of Formula (I), R_1 is hydrogen, halogen, or an optionally substituted C_{1-4} alkoxy. When R_1 is alkoxy, the carbon chain may be optionally substituted, one or more times, independently by hydroxy, halogen, alkoxy, C(O)H, $C(O)_2R_c$, or $C(O)CH_3$ moieties; wherein R_c is hydrogen, C_{1-6} alkyl, aryl, or arylC_{1-4}alkyl. Preferably R_1 is methoxy.

Suitably, for compounds of Formula (I), R_2 is NR_aR_b; R_b is C_{1-10} alkyl, optionally substituted arylC_{1-4} alkyl, optionally substituted heteroaryl C_{1-4} alkyl, optionally substituted C_{3-7} cycloalkyl, or R_a and R_b together with the nitrogen to which they are attached form a 5 to 10 membered ring which optionally contains an additional heteroatom selected from oygen, nitrogen or sulfur; and R_a is hydrogen, or C_{1-4} alkyl, preferably hydrogen, or methyl.

Preferably R_b an optionally substituted benzyl. It is recognized that the alkyl group in the arylalkyl moiety may be branched or straight, such as a methylene or a substituted methylene group , i.e., -CH(CH_3) - aryl. R_a is hydrogen, or C_{1-4} alkyl, preferably hydrogen, or methyl. The optionally substituted aryl moiety of the arylalkyl group, may be substituted one to three times independently by hydroxy, halogen, alkyl or alkoxy.

Suitably, for compounds of Formula (I), m is 1 or 2. Preferably m is 2.

Suitably, for compounds of Formula (I), R_3 is hydrogen, -OC(O)R_5, S(O)n-R_6, or bromo; provided that when R_3 is hydrogen, then R_4 is other than hydrogen. When R_3 is -OC(O)R_5, the R_5 group is suitably C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl, or optionally substituted arylalkyl; preferably R_5 is C_{1-6} alkyl, more preferably methyl.

When R_3 is S(O)n R_6, R_6 is suitably an optionally substituted aryl, or an optionally substituted aryl heteroaryl; and n is 0, or an integer having a value of 1 or 2. When R_6 is heteroaryl, as defined below, it is preferably a triazole, oxadiazole, or tetrazole moiety. When R_6 is aryl, as also defined below, it is preferably a phenyl; the n value is preferably 1 or 2. When R_6 is a heteroaryl, n is preferably 0. The heteroaryl or aryl ring may be optionally substituted one or more times independently by hydroxy, halogen, alkyl or alkoxy, preferably alkyl, more preferably methyl.

Suitably, for compounds of Formula (I), R_4 is hydrogen, bromine, -OC(O)R_5, or $S(O)_n$ R_6, wherein R_5 and R_6 are as defined above in the R_3 group.

Compounds exemplified by Formula (I) include, but are not limited to:
3,4-Dichlorobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

N-3,4-Dichlorobenzyl-N-methyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

N-Methyl-3-iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

S-(-)-Alpha-Methylbenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

Benzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

(R)-Alpha-Methylbenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide

The term "excessive IL-1β convertase activity" is used herein to mean an excessive expression of the protein, or activation of the enzyme.

The term "C_{1-6} alkyl" or "alkyl" is used herein to mean both straight and branched chain radicals of 1 to 6 carbon atoms, unless the chain length is otherwise specified, including, but not limited to, methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *sec*-butyl, *iso*-butyl, *tert*-butyl, and the like.

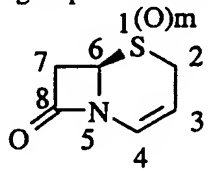
The term "heteroaryl" (on its own or in any combination, such as "heteroaryloxy", or "heteroaryl alkyl") is used herein to mean a 5-10 membered aromatic ring system in which one or more rings contain one or more heteroatoms selected from the group consisting of N, O or S, such as, but not limited, to pyrrole, pyrazole, furan, thiophene, quinoline, isoquinoline, quinazolinyl, pyridine, pyrimidine, oxazole, oxadiazole, tetrazole, thiazole, thiadiazole, triazole, imidazole, benzimidazole, benzothiaphene, benzopyrrole, or benzofuran.

The term "aryl" (on its own or in any combination, such as "aryloxy", or "arylalkyl") is used herein to mean a phenyl and naphthyl ring.

The term "cycloalkyl" is used herein to mean cyclic radicals, preferably of 3 to 7 carbons, including but not limited to cyclopropyl, cyclopentyl, cyclohexyl, and the like.

The term "halo" or "halogens", is used herein to include, unless otherwise specified, chloro, fluoro, bromo and iodo.

For purposes herein the "core" group for Formula (I) is numbered as follows:



The present invention is to the inhibition of ICE and ICE-like proteases by compounds of Formula (I). What is meant by the term "ICE-like proteases" are fragment, homologs, analogs and derivatives of the polypeptides Interleukin-1 β converting enzyme (or convertase). These analogs are structurally related to the ICE family. They generally encode a protein (s) which exhibits high homology to the human ICE over the entire sequence. Preferably, the pentapeptide QACRG is conserved. The ICE like proteases , which may include many natural allelic variants (such as substitutions, deletion or addition of nucleotides) does not substantially alter the function of the encoded polypeptide. That is they retain essentially the same biological function or activity as the ICE protease, although it is recognized that the biological function may be enhanced or reduced activity. The suitable activity is not IL-1β convertase activity, but the ability to induce apoptosis or involved in programmed cell death in some manner. Suitable ICE like proteases encompasses within this invention are those described in PCT US94/07127 filed 23 June 1994, Attorney Docket No.: 325800-184; and in USSN 08/334,251, filed 1 November 1994, Attorney Docket No.: 325800-249 whose disclosures are incorporated herein by reference in their entirety.

The term "blocking or inhibiting, or decreasing the production of IL-1β and/or TNF" as used herein refers to:

a) a decrease of excessive levels, or a down regulation, of the cytokine in a human to normal or sub-normal levels by inhibition of the *in vivo* release of the cytokine; or

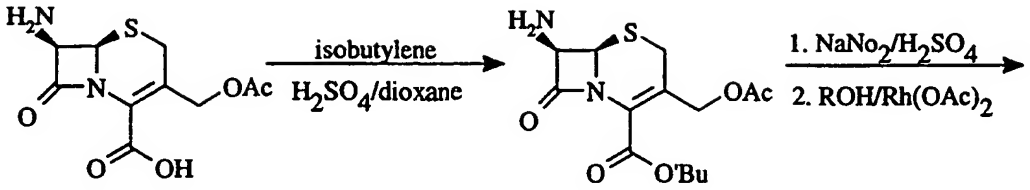
b) a down regulation, at the genomic level, of excessive *in vivo* levels of the cytokine (IL-1 or TNF) in a human to normal or sub-normal levels; or

c) a down regulation, by inhibition of the direct synthesis of the cytokine (IL-1, or TNF) as a postranslational event; or

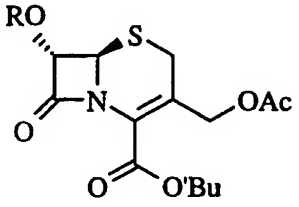
d) a down regulation, at the translational level, of excessive *in vivo* levels of the cytokine (IL-1, or TNF) in a human to normal or sub-normal levels.

The blocking or inhibiting, or decreasing the production of IL-1β and/or TNF is a discovery that the compounds of Formula (I) are inhibitors of the cytokines, IL-1 and TNF is based upon the effects of the compounds of Formulas (I) on the production of the IL-1 and TNF in *in vitro* and *in vivo* assays which are well known and recognized in the art, some of which are described herein.

Compound of the present invention may be synthesized by methods well known in the art, such as those described by the procedures of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513 whose disclosure is incorporated herein by reference. Alternatively, compounds of Formula (I) may be made in accordance with the schemes illustrated below.



I II



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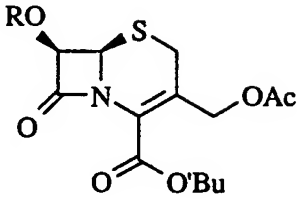

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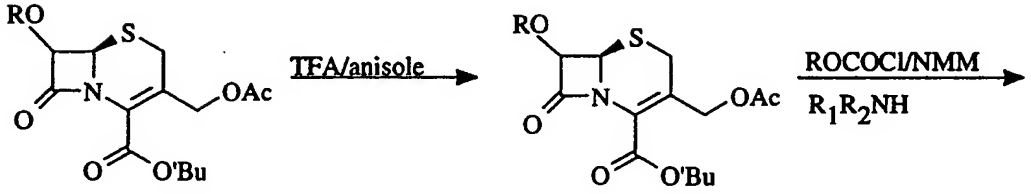
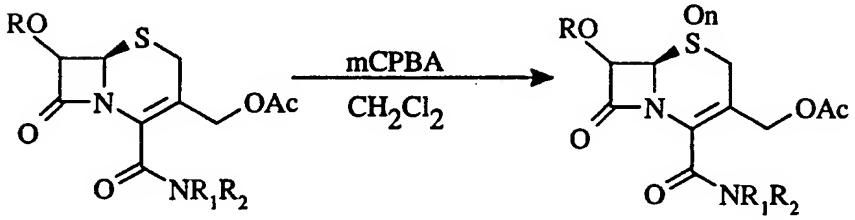


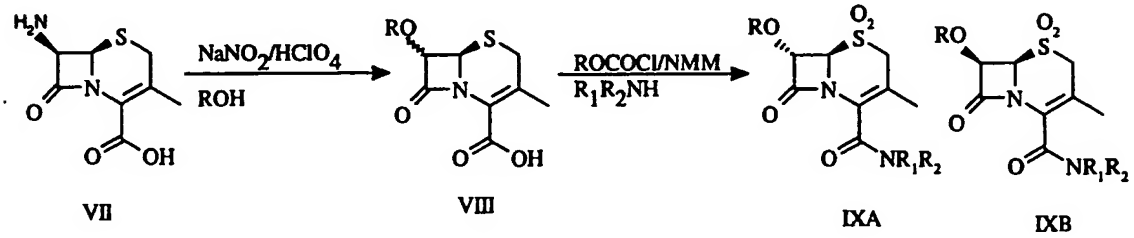
III IV



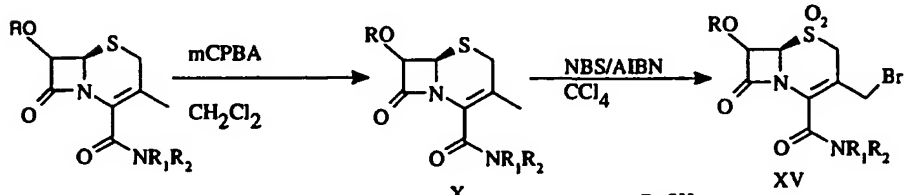
V VI

t-Butyl ester (2-Scheme I) is synthesized by treating commercially available 7-aminocephalosporonic acid (1-Scheme I) with isobutylene and sulfuric acid in dioxane. Following the procedure of Doherty et al. (*J. Med. Chem.* 1990, **33**, 2513-2521, which is incorporated herein by reference), 7-alkoxy substituted 3a-Scheme I and 3b-Scheme I are produced as a separable mixture. Deprotection of 3-Scheme I with trifluoroacetic acid/anisole at 0°C gives the free acid 4-Scheme I. Amine couplings with the activated

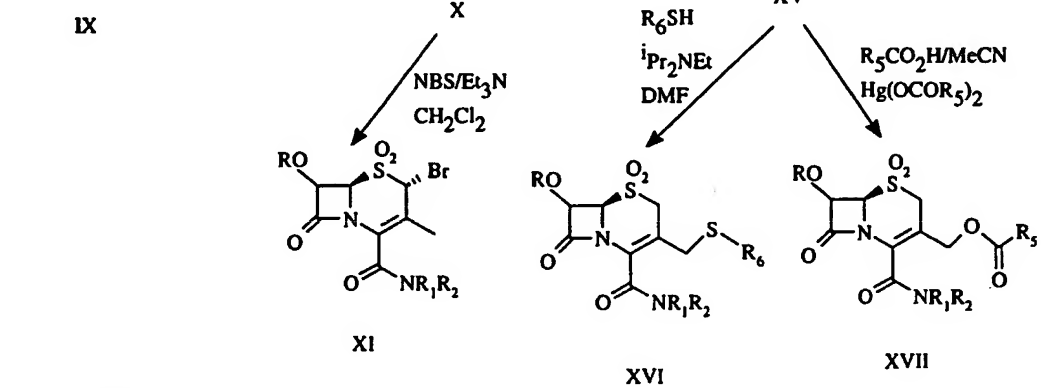
ester of 4-Scheme 1 (alkylchloroformate/N-methylmorpholine) give amides 5-Scheme 1. Oxidation with oxone yields sulfoxides and sulfones 6-Scheme I.



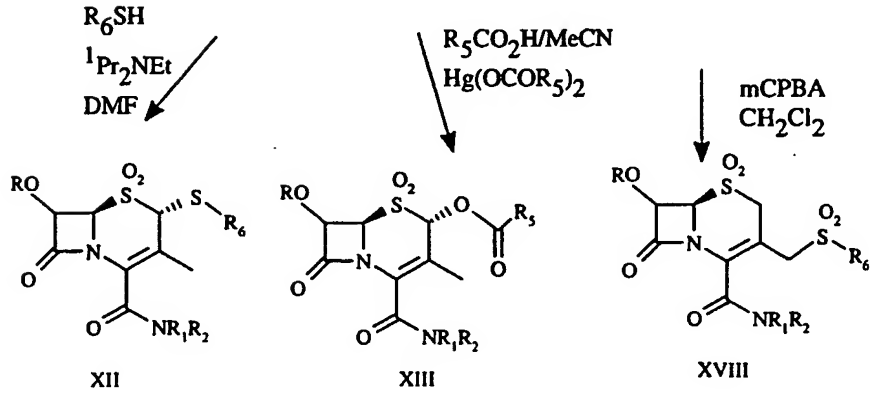
VII VIII IXA IXB



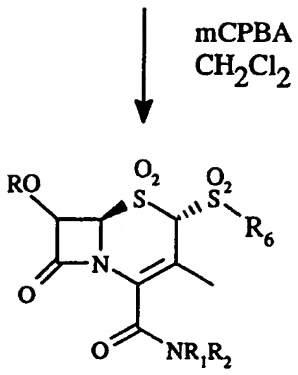
IX X XV



XI XVI XVII



XII XIII XVIII



XIV

Alkoxy derivative 9-Scheme 2 is obtained in one step from 8-Scheme 2 by treatment with $NaNO_2$ and the alcohol in perchloric acid (Alpegiani et al. US 5,254,680, which is incorporated herein by reference). The amide 9-Scheme 2 is formed by amine coupling with acid 8-Scheme 2 by procedures described for 5-Scheme I; oxone oxidation of 9-Scheme 2 yields 10-Scheme 2. The following derivatives can be synthesized according to procedures outlined by Alpegiani et al. *J. Med. Chem.* 1994, 37, 4003-4019, which is incorporated herein by reference: treatment of 10-Scheme 2 with N-bromosuccinimide under anionic conditions gives bromide 11-Scheme 2. Displacement with aromatic thiols or mercuric acetate derivatives yields 12-Scheme I and 13-Scheme 2 respectively. Exposure of 10-Scheme 2 to N-bromosuccinimide under radical conditions gives the 3-bromomethyl derivative 15-Scheme 2; 16-Scheme 2 and 17-Scheme 2 are accessible through displacement of the bromide by aromatic thiols and mercuric acetate derivatives. Sulfones 14-Scheme 2 and 18-Scheme 2 are obtained by oxidation of their corresponding thioethers (12-Scheme 2 and 16-Scheme 2).

SYNTHETIC CHEMISTRY

Without further elaboration, it is believed that one skilled in the art can, using the preceding descriptions, utilize the present invention to its fullest extent. The following examples further illustrate the synthesis of compounds of this invention. The following examples are, therefore, to be construed as merely illustrative and not a limitation of the scope of the present invention in any way.

Temperatures are recorded in degrees centigrade unless otherwise noted.

Example 1

N-3,4-Dichlorobenzyl-(6R, 7S)- 3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide.

a) (6R,7S)-3-Acetoxymethyl-7-methoxy-3-cephem-4-carboxylic Acid

To tert-Butyl (6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxylate (prepared by the procedure of Doherty *et al.*, *J. Med. Chem.*, 1990, 33, 2513) (1.5 g, 4.4 mmol) and anisole (5.0 mL, 45 mmol) was added trifluoroacetic acid (25 mL) at 0 °C under Ar. The solution was stirred for 30 min, and concentrated *in vacuo*. The oil was purified by flash chromatography (silica gel, 50% ethyl acetate/hexanes followed by 90:9:1 methylene chloride/methanol/acetic acid) to yield the title compound as a solid (0.89 g, 70%). MS(ES$^+$) m/e 288 $[M+H]^+$.

b) N-3,4-Dichlorobenzyl-(6R, 7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide

To a stirring solution of the acid of Example 1(a) (0.25 g, 0.88 mmol) in 10 mL of dry THF at -23 °C under Ar was added 4-methylmorpholine (0.15 mL, 1.3 mmol) and ethyl chloroformate (0.13 mL, 1.3 mmol). The resulting mixture was stirred 15 min and then treated with 3,4-dichlorobenzylamine (0.18 ml, 1.3 mmol). The mixture was allowed to warm to 0 °C over 1.5 h.

To the reaction mixture was added 3N HCl (aq) and methylene chloride. The layers were separated and the aqueous layer was extracted twice with methylene chloride. The combined organic layers were dried ($MgSO_4$), filtered, and concentrated *in vacuo* to give a light yellow oil. The oil was purified by flash chromatography (silica gel, 40-50% ethyl acetate/hexanes) to yield the title compound as a light yellow solid (0.28 g, 75%). MS(ES$^+$) m/e 445 $[M+H]^+$.

c) N-3,4-Dichlorobenzyl-(6R, 7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

To the amide of Example 1(b) (0.27 g, 0.62 mmol) in acetonitrile (5 mL) at 0 °C was added a solution of Oxone (0.57 g, 1.9 mmol) in water (5 mL) and the solution was stirred at room temperature for 15 h. To the mixture was added 20% sodium metabisulfite, followed by water and the mixture was extracted twice with methylene chloride. The organic extract was dried ($MgSO_4$), filtered and concentrated *in vacuo* to give an oil. The oil was purified by flash chromatography (silica gel, 50-70% ethyl acetate/hexanes) to yield the title compound (0.17 g, 56%). MS(ES$^+$) m/e 477 $[M+H]^+$.

Example 2

N-Methyl-N-(3,4-dichlorobenzyl)-(6R,7S) -3-acetoxymethyl-7-methoxy -3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting N-methyl-3,4-dichlorobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 491 $[M+H]^+$.

Example 3

N-Methyl-N-(3-iodobenzyl)-(6R,7S)- 3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting N-methyl-3-iodobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 549 $[M+H]^+$.

Example 4

N-(3-Iodobenzyl)-(6R,7S)- 3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 3-iodobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 535 $[M+H]^+$.

Example 5

N-[S-(-)-α-Methylbenzyl]-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting S-(-)-α-methylbenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 423 $[M+H]^+$.

Example 6

N-Benzyl-(6R,7S) -3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting benzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 409 $[M+H]^+$.

Example 7

N-[R-(+)-a-Methylbenzyl]-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting R-(+)-α-methylbenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 423 [M+H]$^+$.

Example 8

N-Piperonyl-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting piperonylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 453 [M+H]$^+$.

Example 9

N-Phenethyl-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting phenethylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 423 [M+H]$^+$.

Example 10

N-(3,4-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-benzyloxycarbonylmethylenoxy-3-cephem-4-carboxamide-1,1-dioxide

Prepared according to the procedure of Example 1 except substituting benzyl glycolate for methanol and concentrating the methylene chloride solution to a small volume before its addition. MS(ES$^+$) m/e 611 [M+H]$^+$.

Example 11

N-(3,4-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-carboxymethylenoxy 3-cephem-4-carboxamide-1,1-dioxide

To the title compound of Example 10 (0.024 g, 0.039 mmol) in ethyl acetate (2 mL) was added a catalytic amount of palladium black. The mixture was stirred under an atmosphere of hydrogen for 2h. The resulting mixture was filtered and concetrated *in vacuo* to yeild the title compound as a white solid (0.020 g, 98%). MS(ES$^+$) m/e 521 [M+H]$^+$.

Example 12

N-(4-Chlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 4-chlorobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 443 $[M+H]^+$.

Example 13

N-(4-Methylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 4-methylbenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 423 $[M+H]^+$.

Example 14

N-(4-Methoxybenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 4-methoxybenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 439 $[M+H]^+$.

Example 15

N-(3-Trifluoromethylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 3-trifluoromethylbenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 477 $[M+H]^+$.

Example 16

N-(4-tert-Butylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 4-tert-butylbenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 465 $[M+H]^+$.

Example 17

N-(2-Methylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 2-methylbenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 423 $[M+H]^+$.

Example 18

N-(2,4-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 2,4-dichlorobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 477 $[M+H]^+$.

Example 19

N-(3,5-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 3,5-dichlorobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 477 $[M+H]^+$.

Example 20

N-(4-Nitrobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 4-nitrobenzylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 454 $[M+H]^+$.

Example 21

N-(2-Naphthylmethylene)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 2-napthylenemethylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 459 $[M+H]^+$.

Example 22

N-(1-Napthylmethylene)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting 1-napthalenemethylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES^-) m/e 457 $[M-H]^-$.

Example 23

N-(3,4-Dichlorobenzyl)-(6R,7R)-7-(2', 2', 2'-trichloroethoxycarboxamido)-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

a) (6R,7R)-7-(2', 2', 2'-trichloroethoxycarboxamido)-3-acetoxymethyl-3-cephem-4-carboxylic acid.

To a suspension of commercially available 7-aminocephalosporonic acid (0.52 g, 1.9 mmol) in a solution of 2.5 ml of water and 5 ml acetone was added sodium bicarbonate ((0.44 g, 5.2 mmol). The resulting solution was treated with 2',2', 2'-trichloroethyl chloroformate in acetone and stirred 16 h. The solution was acidified with 3N HCl and extracted three times with ethyl acetate. The combined organic layers were washed with water, dried ($MgSO_4$), filtered and concentrated *in vacuo* to yield the title compound as a light yellow foam (0.72 g, 86%). ^1H NMR ($CDCl_3$) δ(ppm) 2.1 (3 H, s, CH_3), 3.54 (2 H, q, 2-CH_2), 4.77 (2 H, q, CH_2CCl_3), 5.05 (1 H, d, 6-H), 5.08 (2 H, q, CH_2OAc), 5.68 (1 H, m, 7-H), 6.12 (1 H, d, NH).

b) N-(3,4-Dichlorobenzyl)-(6R,7R)-7-(2', 2', 2'-trichloroethoxycarboxamido)-3-acetoxymethyl-3-cephem-4-carboxamide

To a solution of the acid of Example 23(a) (0.72 g, 1.6 mmol) and 2,4-dinitrophenol (0.30 g, 1.6 mmol) in dry tetrahydrofuran was added a solution of 1,3-dicyclohexylcarbodiimide (0.33 g, 1.6 mmol) in 6 ml methylene chloride. The solution was stirred for 30 min. Filter the mixture and add 3,4-dichlorobenzylamine (0.28 g, 1.6 mmol) to the filtrate. Stir the resulting solution 16 h.

To the solution was added a saturated solution of sodium carbonate and methylene chloride and filter. The layers were then separated and the aqueous layer extracted with methylene chloride. The combined organic layers dried ($MgSO_4$), filtered and concentrated *in vacuo* to give an oil. The oil was purified by flash chromatography (silica gel, 30-60% ethyl acetate/hexanes) to yield the title compound (0.58 g, 60%).%). ^1H NMR ($CDCl_3$) δ(ppm) 2.08 (3 H, s, CH_3), 3.47 (2 H, q, 2-CH_2), 4.5 (2 H, m, CH_2Ph), 4.75 (2 H, q, CH_2CCl_3), 4.86 (2 H, q, CH_2OAc), 5.0 (1 H, d, 6-H), 5.61 (1 H, m, 7-H), 5.98 (1 H, d, $NHCO_2$-), 7.19 (1 H, d, 6-ArH), 7.4 (1 H, d, 5-ArH), 7.48(1H, s, 2-ArH).

c) N-(3,4-Dichlorobenzyl)-(6R,7R)-7-(2', 2', 2'-trichloroethoxycarboxamido)-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1(c), except substituting the amide of Example 23(b) for the amide of Example 1(b), the title compound was prepared. MS(ES$^-$) m/e 634 $[M-H]^-$.

Example 24

N-Cyclohexanemethylene-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting cyclohexanemethylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 415 $[M+H]^+$.

Example 25

N-Furfuryl-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

Following the procedure of Example 1, except substituting furfurylamine for 3,4-dichlorobenzylamine, the title compound was prepared. MS(ES$^+$) m/e 399 $[M+H]^+$.

BIOLOGICAL ASSESSMENTS:

Assay I - DNA Ladder:

The present invention can utilize a model that measures apoptosis, by measuring the production of DNA ladders visualized on agarose gels. The observation of DNA ladders has been a hallmark of the apoptosis response for many years. The model used in our studies is the production of apoptosis in human monocytic HL-60 cells by the anti-cancer ether lipid 1-O-octadecyl-2-O-methyl-*sn*-3-phosphocholine (ET-18-OCH_3) and tumor necrosis factor α (TNF). The production of DNA ladders by ET-18-OCH_3 was recently reported (Mollinedo et al. Biochem. Biophys. Res. Commun., 192: 603-609 (1993)) and confirmed in house. The general method is to treat HL-60 cells with 6 μM ET-18-OCH_3 or 10 units of TNF for 24 hours, followed by extraction of small molecular weight DNA and removal of protein and RNA. The DNA is separated on a agarose gel and visualized with ethidium bromide staining. An internal standard is added to the cells just prior to extraction and preparation of DNA. Drugs are provided to cells 10 minutes prior to the apoptotic insult. This method provides a qualitative assessment of the ability of compounds to inhibit apoptosis.

Cell Conditions

- HL-60 cells (American Type Cell Culture) are grown and kept at log phase in RPMI 1640 w/L-glutamine and 10 % heat inactivated Fetal Bovine Serum (RPMI complete).
- On the day of the experiment, the desired number of cells (for example, 5 x 10^6 cells/treatment group) are resuspend in RPMI complete to give a final cell concentration of approximately 0.5 x 10^6 cells/ml. For each treatment group, 10 mls of cell suspension are placed in a culture flask. Cells are incubated for 2 hours at 37°C.

Exposures:

- For ta ypical expose to ET-18-OCH_3, a 100 mM ET-18-OCH_3 stock solution in $CHCl_3$ is prepared, and diluted in RPMI complete to 600 µM. 100 µl of 600 µM ET-18-OCH_3 is added into 10 ml treatment groups yielding a final concentration of 6 µM. The cell suspensions are then incubated overnight (18 hours). For a typical exposure to TNF, 300 to 3000 units of TNF are added to 10 ml of cell suspension.
- Cells are pretreated with desired agents (ICE compounds, etc.) 10 minutes prior to ET-18-OCH_3 or TNF addition. ICE compounds stocks ware in DMSO. 50 µl of compound or DMSO vehicle are added to the 10 ml treatment groups yielding a final concentration of compound and 0.5 % DMSO.

DNA Extraction:

- Cells are spun (400 x g, 5 min) and washed 2x in 10 mLs PBS.
- Cells are lysed by resuspending them into 200 µL of cold, sterile detergent buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% Triton X-100) and transferring the approximately 250 µL volume to sterile, 1.5 mL eppendorf tubes on ice. Tubes are incubated for 30 min. at 4°C, with mild shaking.
- Tubes are spun in a Microfuge for 15 min., the supernatant collected, taking care to avoid cellular debris.
- The supernatants are incubated with 75 µg/mL RNAseA for 1 hr at 37°C than incubated with 200 µg/mL ProteinaseK and 0.5 % SDS [final] for 1 hr at 37°C.
- Ten µl of a 300 bp DNA is added as an internal standard to observe extraction efficiency.
- Supernatants are extracted twice with equal volume (200-300 µL) of cold, buffer saturated phenol (add phenol, vortex 15 seconds, microfuge 2 min., collecting the top aqueous layer, avoiding the organic waste in between the two phases), once with 200 µL Phenol/Chloroform/Isoamyl alcohol 25:24:1 (v/v) and once with 100 µL Chloroform (100µL/sample is retrieved).

- Add 10 µL of sterile 3M NaCl (300 mM [final]) to the 100 µL sample and 200 µL of cold ethanol, vortex well and let stand overnight at -20°C.
- Samples are spun (Microfuge) 15 min and all but 25 µl of the ethanol is carefully removed. The DNA pellets are dried and resuspended in 30 µL of sterile 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA and 10 µL of gel loading Buffer. Load 20 µl/well.
- A DNA standard (for example, Sigma # D 5042, 123 bp ladder) is run on each gel.
- Samples are run on 1-2 % agarose gel with TBE buffer (5X TBE = 54 g Tris Base, 27.5 g Boric acid, 20ml 0.5 M EDTA, pH 8.0) with ethidium bromide added, for example for 90 - 120 min at 100 V, 50 mA.
- The resulting gels are visualized under UV light and the results recorded in a captured image.

Assay II: Inhibition of ICE

Source of Enzyme

Human ICE was cloned and expressed in *E. coli* as its inactive precursor (p45) bearing a hexa-His flag on its *amino*-terminal end. Following harvesting, the cells were lysed, centrifuged, and the pellet containing the p45 solubilized with phosphate buffered 7 M urea at pH 7.5. The flagged p45 was applied to a Ni-nitrilo-acetic acid column, washed, and eluted with 300 mM imidazole. This yielded a highly enriched proenzyme preparation (³90% pure p45). Catalytic autoproteolytic activation to p10/p20 dimer was achieved by concentrating the p45 on a Centricon ultrafiltration membrane (Amicon) at 10 _C for several hours. The formation of the catalytic subunits (p10 and p20) in activated samples was demonstrated by correlating time-dependent generation of ICE activity with p10/p20 signals in Western blots and by reversed-phase HPLC. Formation of authentic p10 and p20 was also confirmed by *N*-terminal sequence and MALD-mass spectral analyses of samples purified by reversed-phase HPLC. The activated enzyme was stored frozen at –80 _C.

Assay Protocol

ICE was assayed at 25 _C using the fluorogenic tetrapeptide substrate *N*-acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartyl-7-amido-4-methylcoumarin (Ac-YVAD-AMC). The assays were conducted at pH 7.5 in a buffered system containing 25 mM Hepes, 10% sucrose, 0.1% CHAPS, and 2 mM DTT. The concentration of substrate was fixed at 25 uM. Fluorescence of the liberated 7-amino-4-methylcoumarin was continuously monitored at 460 nm following excitation at 335 nm.

Compound Testing

Compounds of Formula (I) were tested at a single dose of 100 uM following a 30 to 60-min preincubation with enzyme. The assay was initiated by the addition of 25 uM substrate (Ac-**YVAD**-AMC) and activity was monitored as described above.

A representative compound of Formula (I), Example 2 demonstrated positive inhibitory activity in this assay.

Assay III: Inhibition of ICE

ICE was assayed at 25 _C in 96-well plates using the fluorogenic tetrapeptide substrate *N*-acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartyl-7-amido-4-methylcoumarin (Ac-**YVAD**-AMC). The assays were conducted at pH 7.5 in a buffered system containing 25 mM Hepes, 10% sucrose, 0.1% CHAPS, and 20-50 uM DTT. The concentration of substrate was fixed at 20 uM. Fluorescence of the liberated 7-amino-4-methylcoumarin was continuously monitored at 460 nm following excitation at 360 nm.

Compound Testing

Compounds were tested at a single dose of 50 to 100 uM. Activity was monitored as described above over a 30 to 60-minute time period following the simultaneous addition of substrate and inhibitor to initiate the reaction. The progress curves thus generated were fit by computer to Eq. 1 in order to assess potency and time-dependency:

$$v = \frac{(V_0(1 - e^{-k_{obs}t})}{k_{obs}} \qquad (1)$$

Representative compounds of formula (I) have demonstrated positive inhibitory activity in the above noted assay:

3,4-Dichlorobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

N-Methyl-3-iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

3-Iodobenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

The compound S-(-)-Alpha-Methylbenzyl-(6R,7S)-7-methoxy-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide demonstrated a 0% inhibitory activity in this assay for undetermined reasons.

METHODS OF TREATMENT

For therapeutic use the compounds of the present invention will generally be administered in a standard pharmaceutical composition obtained by admixture with a pharmaceutical carrier or diluent selected with regard to the intended route of administration and standard pharmaceutical practice. For example, they may be administered orally in the form of tablets containing such excipients as starch or lactose, or in capsule, ovules or lozenges either alone or in admixture with excipients, or in the form of elixirs or suspensions containing flavouring or colouring agents. They may be injected parenterally, for example, intravenously, intramuscularly or subcutaneously. For parenteral administration, they are best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The choice of form for administration as well as effective dosages will vary depending, inter alia, on the condition being treated. The choice of mode of administration and dosage is within the skill of the art.

The compounds of the present invention, particularly those noted herein or their pharmaceutically acceptable salts which are active when given orally, can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and lozenges.

A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerin, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule. Preferably the composition is in unit dose form such as a tablet or capsule.

Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilized and then reconstituted with a suitable solvent just prior to administration.

A typical suppository formulation comprises a compound or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding

and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

The pharmaceutically acceptable compounds of the invention will normally be administered to a subject in a daily dosage regimen. For a patient this may be, for example, from about .001 to about 100mg/kg, preferably from about 0.001 to about 10mg/kg animal body weight. A daily dose, for a larger mammal is preferably from about 1 mg to about 1000 mg, preferably between 1 mg and 500 mg or a pharmaceutically acceptable salt thereof, calculated as the free base, the compound being administered 1 to 4 times per day. Unit dosage forms may contain from aobut 25μg to about 500mg of the compound.

There are many diseases and conditions in which dysregulation of apoptosis plays an important role. All of these conditions involve undesired, deleterious loss of specific cells with resulting pathological consequences.

Bone remodeling involves the initial resorption by osteoclasts, followed by bone formation by osteoblasts. Recently, there have been a number of reports of apoptotic events occurring during this process. Apoptotic events have been observed in both the bone forming and bone resorbing cells *in vitro* and indeed at the sites of these remodeling units *in vivo.*

Apoptosis has been suggested as one of the possible mechanisms of osteoclast disappearance from reversal sites between resorption and formation. TGF-ß1 induces apoptosis (approx. 30%) in osteoclasts of murine bone marrow cultures grown for 6 days *in vitro* . (Hughes, et al., *J. Bone Min. Res.* 9 , S138 (1994)). The anti-resorptive bisphosphonates (clodronate, pamidronate or residronate) promote apoptosis in mouse osteoclasts *in vitro* and *in vivo* . (Hughes, et al., supra at S347). M-CSF, which has previously been found to be essential for osteoclast formation can suppress apoptosis, suggesting not only that maintenance of osteoclast populations, but also that formation of these multinucleated cells may be determined by apoptosis events. (Fuller, et al., *J. Bone Min. Res.* 8 , S384 (1993); Perkins, et al., *J. Bone Min. Res.* 8 , S390 (1993)). Local injections of IL-1 over the calvaria of mice once daily for 3 days induces intense and aggressive remodeling. (Wright, et al., *J. Bone Min. Res.* 9 , S174 (1994)). In these studies, 1% of osteoclasts were apoptotic 1 day after treatment, which increased 3 days later to 10%. A high percentage (95%) of these apoptotic osteoclasts were at the reversal site. This data suggests that ICE or ICE-like homologues are functionally very important in osteoclast apoptosis.

Therefore, one aspect of the present invention is the promotion of apoptosis in osteoclasts as a novel therapy for inhibiting resorption in diseases of excessive bone loss, such as osteoporosis, using compounds of Formula (I) as defined herein.

Apoptosis can been induced by low serum in highly differentiated rat osteoblast-like (Ros 17/2.8) cells (Ihbe, et al., (1994) *J. Bone Min. Res.* 9, S167)). This was associated with a temporal loss of osteoblast phenotype, suggesting that maintenance of lineage specific gene expression and apoptosis are physiologically linked. Fetal rat calvaria derived osteoblasts grown *in vitro* undergo apoptosis and this is localized to areas of nodule formation as indicated by *in situ* end-labeling of fragmented DNA. (Lynch, et al., (1994) *J. Bone Min. Res.* 9, S352). It has been shown that the immediate early genes c-fos and c-jun are expressed prior to apoptosis; c-fos and c-jun-Lac Z transgenic mice show constitutive expression of these transcription factors in very few tissues, one of which is bone (Smeyne, et al., (1992) *Neuron.* 8, 13-23; and Morgan, J. (1993) Apoptotic Cell Death: Functions and Mechanisms. Cold Spring Harbor 13-15th October). Apoptosis was observed in these animals in the epiphyseal growth plate and chondrogenic zones as the petula ligament calcifies. Chondrogenic apoptosis has also been observed in PTHRP-less mice and these transgenics exhibit abnormal endochondral bone formation (Lee, et al., (1994) *J. Bone Min. Res.* 9, S159). A very recent paper examined a human osteosarcoma cell line which undergoes spontaneous apoptosis. Using this cell line, LAP-4, but not ICE, could be detected and *in vitro* apoptosis could be blocked by inhibition or depletion of LAP-4 (Nicholson, et al., (1995) *Nature* 376, 37-43). Thus, apoptosis may play a role in loss of osteoblasts and chondrocytes and inhibition of apoptosis could provide a mechanism to enhance bone formation.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to enhance bone formation using compounds of Formula (I) as defined herein.

Osteoarthritits (OA) is a degenerative disease characterized by progressive erosion of articular cartilage. Chondrocytes are the single cell-type found in articular cartilage and perturbations in metabolism of these cells may be involved in the pathogenesis of OA. Injury to cartilage initiates a specific reparative response which involves an increase in the production of proteoglycan and collagen in an attempt to reestablish normal matrix homeostasis. However, with the progress of the disease, the 3-dimensional collagen network is disrupted and cell death of chondrocytes occurs in OA lesions (Malemud, et al.: Regulation of chondrocytes in osteoarthritis. In: Adolphe, M. ed. Biological Regulation of Chondrocytes. Boca Raton:CRC Press, 1992, 295-319). It has been shown that in OA, chondrocytes adjacent to cartilage defects express high levels of bcl-2 (Erlacher, et al., (1995) *J. of Rheumatology*, 926-931). This

represents an attempt to protect chondrocytes from apoptosis induced by the disease process.

Protection of chondrocytes during early degenerative changes in cartilage by inhibition of apoptosis may provide a novel therapeutic approach to this common disease.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat osteoarthritis, using compounds of Formula (I) as defined herein.

Recent evidence shows that chronic, degenerative conditions of the liver are linked to hepatocellular apoptosis. These conditions include chemical-, infectious- and immune/inflammatory-induced hepatocellular degeneration. Apoptosis of liver cells has been observed in liver degenerative states induced by a variety of chemical agents, including acetaminophen (Ray, et a l.,(1993) *FASEB. J.* **7**, 453-463), cocaine (Cascales, et al., (1994) *Hepatology* **20**, 992-1001) and ethanol (Baroni, etal., (1994) *J. Hepatol.* **20**, 508-513). Infectious agents and their chemical components that have been shown to induce apoptosis include hepatitis ((Hiramatsu, et al., (1994) *Hepatology* **19**, 1354-1359; Mita, et al., (1994) *Biochem. Biophys. Res. Commun.* **204**, 468-474)), tumor necrosis factor and endotoxin . (Leist, et al., (1995) *J. Immunol.* **154**, 1307-1316; and Decker, K. (1993) *Gastroenterology* **28(S4)**, 20-25). Stimulation of immune / inflammatory responses by mechanisms such as allograft transplantation and hypoxia followed by reperfusion have been shown to induce apoptosis of hepatocytes (Krams, et al., (1995) *Transplant. Proc.* **27**, 466-467). Together, this evidence supports that hepatocellular apoptosis is central to degenerative liver diseases.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat degenerative liver diseases., using compounds of Formula (I) as defined herein.

Apoptosis is recognized as a fundamental process within the immune system where cell death shapes the immune system and effects immune functions. Apoptosis also is implicated in viral diseases (e.g AIDS). Recent reports indicate that HIV infection may produce an excess of apoptosis, contributing to the loss of $CD4^+$ T cells. Of additional interest is the observation that APO-1/Fas shares sequence homology with HIV-1 gp120.

Therefore, another aspect of the present invention is the inhibition of apoptosis as a novel therapy to treat viral diseases, using compounds of Formula (I) as defined herein.

Additional therapeutic directions and other indications in which inhibition of apoptotic cysteine proteases is of therapeutic utility, along with relevant citations in support of the involvement for apoptosis in each indication, are presented below in Table 1.

Table 1: Therapeutic Indications Related to Apoptosis

Indication	Citations
Ischemia / reperfusion	Barr et al., (1994) *BioTechnology* **12**, 487-493; Thompson, C. B. (1995) *Science* **267**, 1456-1462
Stroke	Barr et al supra; and Thompson, C., supra
Polycystic kidney disease	Barr et al., supra; and Mondain, et al., (1995) *ORL J. Otorhinolaryngol. Relat. Spec.* **57**, 28-32
Glomerulo-nephritis	Barr et al., supra
Osteoporosis	Lynch et al., (1994) *J. Bone Min. Res.* **9**, S352; Nicholson et al., (1995) *Nature* **376**, 37-43
Erythropoiesis / Aplastic anemia	Thompson, C., supra; Koury et al., (1990) *Science* **248**, 378-381
Chronic liver degeneration	Thompson, C., supra; Mountz et al., 1994) *Arthritis Rheum.* **37**, 1415-1420; Goldin et al., (1993) *Am. J. Pathol.* **171**, 73-76
T-cell death	Thompson, C., supra; Ameison et al., (1995) *Trends Cell Biol.* **5**, 27-32
Osteoarthritis - chondrocytes	Ishizaki et al., (1994) *J. Cell Biol.* **126**, 1069-1077; Blanco et al., (1995) *Am. J. Pathol.* **146**, 75-85
Male pattern baldness	Mondain et al., supra; Seiberg et al., (1995) *J. Invest. Dermatol.* **104**, 78-82; Tamada et al., (1994) *Br. J. Dermatol.* **131**, 521-524

Alzheimer's disease	Savill, J.,(1994) *Eur. J. Clin. Invest.* **24**, 715-723; Su et al., (1994) *Neuroreport* **5**, 2529-2533; Johnson, E., (1994) *Neurobiol. Aging* **15 Suppl. 2**, S187-S189
Parkinson's disease	Savill, J., supra; Thompson, C., supra
Type I diabetes	Barr et al., supra

The IL-1 and TNF inhibiting effects of compounds of the present invention are determined by the following *in vitro* assays:

Interleukin - 1 (IL-1)

Human peripheral blood monocytes are isolated and purified from either fresh blood preparations from volunteer donors, or from blood bank buffy coats, according to the procedure of Colotta *et al*, J Immunol, **132**, 936 (1984). These monocytes ($1x10^6$) are plated in 24-well plates at a concentration of 1-2 million/ml per well. The cells are allowed to adhere for 2 hours, after which time non-adherent cells are removed by gentle washing. Test compounds are then added to the cells for about 1hour before the addition of lipopolysaccharide (50 ng/ml), and the cultures are incubated at 37°C for an additional 24 hours. At the end of this period, culture super-natants are removed and clarified of cells and all debris. Culture supernatants are then immediately assayed for IL-1 biological activity, either by the method of Simon *et al.*, J. Immunol. Methods, **84**, 85, (1985) (based on ability of IL-1 to stimulate a Interleukin 2 producing cell line (EL-4) to secrete IL-2, in concert with A23187 ionophore) or the method of Lee *et al.*, J. ImmunoTherapy, **6** (1), 1-12 (1990) (ELISA assay).

Tumour Necrosis Factor (TNF):

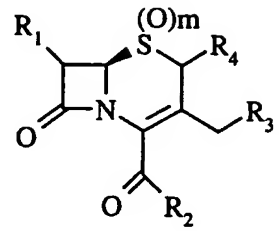
Human peripheral blood monocytes are isolated and purified from either blood bank buffy coats or platelet pheresis residues, according to the procedure of Colotta, R. *et al.*, J Immunol, **132**(2), 936 (1984). The monocytes are plated at a density of $1x10^6$ cells/ml medium/well in 24-well multi-dishes. The cells are allowed to adhere for 1 hour after which time the supernatant is aspirated and fresh medium (1ml, RPMI-1640, Whitaker Biomedical Products, Whitaker, CA) containing 1% fetal calf serum plus penicillin and streptomycin (10 units/ml) added. The cells are incubated for 45 minutes in the presence or absence of a test compound at 1nM-10mM dose ranges (compounds are solubilized in dimethyl sulfoxide/ethanol, such that the final solvent concentration in the culture medium is 0.5%

dimethyl sulfoxide/0.5% ethanol). Bacterial lipopoly-saccharide (*E. coli* 055:B5 [LPS] from Sigma Chemicals Co.) is then added (100 ng/ml in 10 ml phosphate buffered saline) and cultures incubated for 16-18 hours at 37°C in a 5% CO_2 incubator. At the end of the incubation period, culture supernatants are removed from the cells, centrifuged at 3000 rpm to remove cell debris. The supernatant is then assayed for TNF activity using either a radio-immuno or an ELISA assay, as described in WO 92/10190 and by Becker *et al.*, J Immunol, 1991, **147**, 4307.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the are can, using the preceding description, utilize the present invention to its fullest extent. Therefore the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.

What is claimed is:

1. A compound of the formula:



(I)

wherein

R_1 is hydrogen, halogen, or an optionally substituted alkoxy;

R_2 is NR_aR_b;

R_a is hydrogen, or C_{1-4} alkyl;

R_b is C_{1-10} alkyl, optionally substituted arylC_{1-4} alkyl, optionally substituted heteroaryl C_{1-4} alkyl, optionally substituted C_{3-7} cycloalkyl, or R_a and R_b together with the nitrogen to which they are attached form a 5 to 10 membered ring which optionally contains an additional heteroatom selected from oygen, nitrogen or sulfur;

R_3 is hydrogen, -OC(O)R_5, or S(O)n R_6, or bromo;

R_4 is hydrogen, -OC(O)R_5, bromo or S(O)n R_6, provided that when R_3 is hydrogen, R_4 is other than hydrogen;

R_5 is C_{1-6} alkyl, C_{3-7} cycloalkyl, optionally substituted aryl or optionally substtituted arylalkyl;

R_6 is optionally substituted aryl, or optionally substituted heteroaryl;

m is an integer having a value of 1 or 2;

n is 0, or an integer having a value of 1 or 2;

or a pharmaceutically acceptable salt thereof.

2. The compound according to Claim 1 wherein the R_1 moiety is an optionally substituted alkoxy moiety.

3. The compound according to Claim 2 wherein the R_1 alkoxy is methoxy or 2-hydroxyethoxy.

4. The compound according to Claim 1 wherein R_b is optionally substituted benzyl.

5. The compound according to Claim 4 wherein R_a is methyl.

6. The compound according to Claim 1 wherein m is 2.

7. The compound according to Claim 1 wherein R3 is S(O)n R6.

8. The compound according to Claim 7 wherein R6 is a heteroaryl which is an optionally subsituted tetrazole, triazole, or oxadiazole.

9. The compound according to Claim 1 wherein R3 is hydrogen.

10. The compound according to Claim 1 which is:
N-3,4-Dichlorobenzyl-(6R, 7S)- 3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide.
N-Methyl-N-(3,4-dichlorobenzyl)-(6R,7S) -3-acetoxymethyl-7-methoxy -3-cephem-4-carboxamide-1,1-dioxide
N-Methyl-N-(3-iodobenzyl)-(6R,7S)- 3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-(3-Iodobenzyl)-(6R,7S)- 3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-[S-(-)-α-Methylbenzyl]-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-Benzyl-(6R,7S) -3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-[R-(+)-a-Methylbenzyl]-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-Piperonyl-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-Phenethyl-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-(3,4-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-benzyloxycarbonylmethylenoxy-3-cephem-4-carboxamide-1,1-dioxide
N-(3,4-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-carboxymethylenoxy 3-cephem-4-carboxamide-1,1-dioxide
N-(4-Chlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide
N-(4-Methylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(4-Methoxybenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(3-Trifluoromethylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(4-tert-Butylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(2-Methylbenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(2,4-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(3,5-Dichlorobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(4-Nitrobenzyl)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(2-Naphthylmethylene)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(1-Napthylmethylene)-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-(3,4-Dichlorobenzyl)-(6R,7R)-7-(2', 2', 2'-trichloroethoxycarboxamido)-3-acetoxymethyl-3-cephem-4-carboxamide-1,1-dioxide

N-Cyclohexanemethylene-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

N-Furfuryl-(6R,7S)-3-acetoxymethyl-7-methoxy-3-cephem-4-carboxamide-1,1-dioxide

or a pharmaceutically acceptable salt thereof

11. A pharmaceutical composition comprising a compound according to Claim 1 and a pharmaceutically acceptable carrier or diluent.

12. A method of blocking excess or inappropriate apoptosis in a mammal in need of such treatment which method comprises administering to said mammal or human an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

13. The method according to Claim 12 wherein the excessive or inappropriate apoptosis occurs in Alzheimer disease.

14. The method according to Claim 12 wherein the excessive or inappropriate apoptosis occurs in viral infections.

15. The method according to Claim 12 wherein the excessive or inappropriate apoptosis occurs during infarction or reperfusion injury.

16. The method according to Claim 12 wherein the excessive or inappropriate apoptosis occurs during ischemia.

17. The method according to Claim 12 wherein the excessive or inappropriate apoptosis results in excessive bone loss.

18. The method according to Claim 12 wherein the excessive or inappropriate apoptosis results in the disease of osteoarthritis.

19. The method according to Claim 12 wherein the excessive or inappropriate apoptosis results in hepatocellular degeneration.

20. A method for the treatment of diseases or disorders associated with excessive IL-1β convertase activity, in a mammal in need thereof, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

21. A method of blocking or decreasing the production of IL-1β and/or TNF, in a mammal in need of such treatment, which method comprises administering to said mammal an effective amount of a compound of Formula (I), or a pharmaceutically acceptable salt thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13867

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/545; C07D 501/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 540-223, 224, 226, 222, 230, 229, 228, 215;
514-200, 202, 203, 204, 207, 208, 209

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN
Chemical Abstracts by Structure

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Chem. Abstr; vol. 121. 10 October 1994 (Columbus, Ohio, USA); Davies et al., "Substituted cephalosporin sulfone formations useful in the treatment of Leukemia leukemia" G.B. 2,266,525; 03 November 1993,see entire abstract.	1-21 / 1-21
X -- Y	Chem. Abstract, vol. 113,24 September 1990, (Columbus, Ohio, USA) Fin Ke et al. "Inhibition of human leukocyte elastase", J. Med. Chem. 33(9), 2522-8 (1990), see entire abstract.	1-21 / 1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 DECEMBER 1996	Date of mailing of the international search report 10 JAN 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN M. FORD aco Telephone No. (703) 308-1235

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/13867

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

540-223, 224, 226, 222, 230, 229, 228, 215;
514-200, 202, 203, 204, 207, 208, 209